Isolation of Sulfonamides from Fortified Chicken Tissues with Supercritical CO_2 and In-Line Adsorption

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Abstract

Improved recoveries and detectability of three sulfonamides from chicken tissues by supercritical fluid extraction (SFE), without modifiers, using an in-line adsorption trap, are reported. Following SFE, the analytes are recovered from neutral alumina with the HPLC mobile phase. Samples are injected directly onto high-performance liquid chromatographic columns without post-extraction cleanup. Mean recoveries of sulfamethazine, sulfadimethoxine, and sulfaquinoxaline from liver, breast tissue, and thigh muscle are 89, 95, and 77%, respectively. The analytes are detectable at less than 100 ppb with a minimum of background interference.

Introduction

The Food Safety and Inspection Service (FSIS), U.S. Department of Agriculture, is responsible for screening edible animal tissues for violations of U.S. Federal Regulations regarding veterinary drug residues in these products. Among the drug classes under surveillance are the sulfonamides, which are widely used for the prevention and treatment of disease in farm stock. Current methodology used by FSIS to monitor for sulfa drug residues in edible tissues employs solvent extraction techniques. This method uses significant quantities of ethyl acetate and dichloromethane to isolate sulfonamides, followed by thin-layer chromatographic analysis for quantitation (1). The Environmental Protection Agency has decreed, however, that by 1995 government funded contracts and selected federal laboratories, including FSIS, must reduce their use of specified solvents (e.g., dichloromethane) by 50% (2).

Because of the need to reduce the amount of solvents used in analytical methods and to improve their efficiency, we began a program to determine if supercritical fluid extraction (SFE) could be used to supplant some of the methods currently used to isolate veterinary drugs from meat and meat products. Our initial work in this area led to the development of a supercritical fluid restrictor–collector interface that allowed for the off-line collection of analytes on commercial solid-phase extraction (SPE) columns (3). Preliminary results with a class of anti-coccidial compounds, the nitrobenzamides, was encouraging. Therefore, we extended our studies to determine the feasibility of using SFE for the isolation of more polar residues (e.g., sulfonamides) from edible tissues in lieu of solvent extraction.

Only limited studies have been published on the use of supercritical fluids in the extraction of sulfonamides from tissue (4,5). Although fair to good recoveries of individual analytes were reported, the instrumental limitations and SFE parameters that were employed necessitated the use of up to 25% methanol as a CO_2 modifier to obtain these results. This paper reports observations and results of our efforts to develop a procedure for the quantitative extraction and recovery of sulfonamides from edible tissues using supercritical CO_2 without modifiers and compares the role of in-line versus off-line trapping of extracted analytes to attain this goal.

Experimental

Materials

Hydromatrix (Celite 566) obtained from Varian Sample Preparation Products (Part No. 0019-8003) (Harbor City, CA) was sieved (#35 mesh) to remove fines. Neutral alumina (Brockman #1, 80–200 mesh) was purchased from Fisher Scientific (Malvern, PA). SFC-grade carbon dioxide was purchased from Scott Specialty Gas (Plumsteadville, PA). Methanol (MeOH), Burdick and Jackson high-purity solvent, was a product of Baxter Health Care (Muskegon, MI). HPLC-grade *N,N*-dimethylformamide (DMF), 40% aqueous tetrabutylammonium hydroxide (TBAH), sulfamethazine (SMZ), and sulfadimethoxine (SDM) were obtained from Sigma Chemical (St. Louis, MO). Sulfaquinoxaline (SQX) was purchased from Pfaltz and Bauer (Waterbury, CT). Standard solutions containing 0.2 µg/µL and 0.02 µg/µL of SMZ (MeOH), SDM (MeOH), and SQX (DMF) were prepared and were used to fortify tissue samples.

Procedure

For sample preparation, 1.0 g of cubed, frozen, ground tissue was fortified with 5 µL of the sulfa drug standard solution by depositing the solution on the surface of the tissue. Fortified tissue samples were held at –10°C for 1 h and then added in a frozen

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state to 2.0 g Hydromatrix contained in a 50-mL beaker. Following partial thawing, the tissue was ground into the Hydromatrix with a metal spatula until the tissue was dispersed throughout.

Packing of SFE vessels

Off-line recovery. A high-pressure (10,000 psi) extraction vessel (approximately 26-mL volume) was assembled from a nipple (CNLX1208) and two couplings (20F12463) obtained from Autoclave Engineers (Erie, PA). Each coupling was fitted with a 40-mesh i.d. stainless steel wire cloth disk (Small Parts; Miami, FL), which served as frits in the assembled vessel. The nipple was filled in the following order: a plug of polypropylene wool (Aldrich; Milwaukee, WI); 5.0 g Hydromatrix; polypropylene wool; the tissue–Hydromatrix mixture; and a plug of polypropylene wool. Individual matrix–polypropylene wool additions were packed tightly using a stainless steel tamping rod (assembled in-house) designed to fit snugly in the nipple.

In-line recovery. To the nipple the following was added in order: polypropylene wool; 3.5 g Hydromatrix; a layer of polypropylene wool; tissue–Hydromatrix mixture; polypropylene wool; 2.0 g neutral Al_2O_3; and finally a plug of polypropylene wool. All of it was packed tightly in the manner described above. The packed extraction vessel containing the in-line alumina trap is depicted in Figure 1. All assembled vessels containing sample matrix were stored at 4°C until they were ready to be connected to the SFE apparatus.

SFE procedure

The supercritical fluid extractor was fabricated in this laboratory as previously described (3). The apparatus maintained back pressure by means of a micrometering valve (Part No. 10VRMM2812, Autoclave Engineers) that was encased in a temperature-controlled aluminum block. SPE columns were attached directly to this valve through a specially designed interface (3). Following installation of the extraction vessel in the SFE apparatus, the closed system was pressurized to 9,000 psi using a gas booster pump (Model AGT-62/152C, Haskell Engineering; Burbank, CA), and the oven was heated to 40°C. The micrometering valve had been preheated to 110°C prior to pressurization. After the extraction vessel reached the set point temperature, the pressure was adjusted to 10,000 psi (density: 1.042 g/mL). To equilibrate the fluid system, a 4-min static holding period was employed during each experiment. During this period, a 6-mL SPE column (Applied Separations; Allentown, PA) containing 2.0 g neutral alumina that was packed in-house was attached to the micrometering valve through the interface. The SPE column in turn was connected to a flow meter (Model 110, McMillian; Georgetown, TX) and a gas totalizer (Model DTM-115; American Meter; Philadelphia, PA). After the holding period had elapsed, a shut-off valve was opened, and the flow was directed to the micrometering valve. The valve was adjusted to give a flow rate of 3 L/min of depressurized CO_2 through the SPE column and was maintained at this rate for 40 min (120 L expanded gas). This procedure was used for both in-line and off-line analyte trapping experiments.

Sulfonamide recovery from SPE columns and chromatography

Off-line SPE. At the conclusion of the extraction, flow to the micrometering valve was stopped, and the SPE column was removed from the interface. Residual material remaining on the discharge tube of the micrometering valve was washed into the SPE column with approximately 0.25 mL of the HPLC mobile phase. The extracted material clinging to the sides of the column was washed onto the alumina with two 0.5-mL portions of HPLC mobile phase. The sulfonamides were eluted from the alumina with the mobile phase collecting the first 2–4 mL of eluent. Then 100 µL of eluent was injected onto the HPLC column.

In-line SPE. The extraction vessel, following depressurization, was removed from the SFE apparatus. The upper coupling and top layer of polypropylene wool were removed from the vessel, which was maintained in a vertical position throughout this procedure. The alumina sorbent bed was recovered by pouring it into a 6-mL SPE column containing 0.5 g neutral alumina sandwiched between 20-µm polypropylene frits. The alumina was compacted by tapping the sides and top of the SPE column with a spatula, and then a 0.5-cm layer of sand was placed on top. Analytes were recovered by elution with the HPLC mobile phase under slight pressure; the first 2 mL of eluent was collected. Then 100 µL of eluent was injected into the HPLC system.

HPLC analysis. Analyses were performed with an Isco LC-5000 syringe pump (Isco; Lincoln, NE) and Rheodyne Model 7125 injector (Berkley, CA) connected to a 25-cm × 4.6-mm i.d. Supelcosil LC-18 column (5-µm film) (Supelco; Bellefonte, PA). Sulfonamide detection was accomplished at 265 nm using an Applied Biosystems Model 1000S diode array detector (Foster City, CA). The HPLC mobile phase was 65% 0.05M phosphate buffer containing 0.1% TBAH (final pH 7.2) and 35% MeOH at a flow rate of 0.9 mL/min. Chromatograms were recorded on a Hewlett-Packard Model 3396A integrator (Valley Forge, PA) and Fisher Recordall Series 5000 recorder at 10 mV full scale. Quantitation

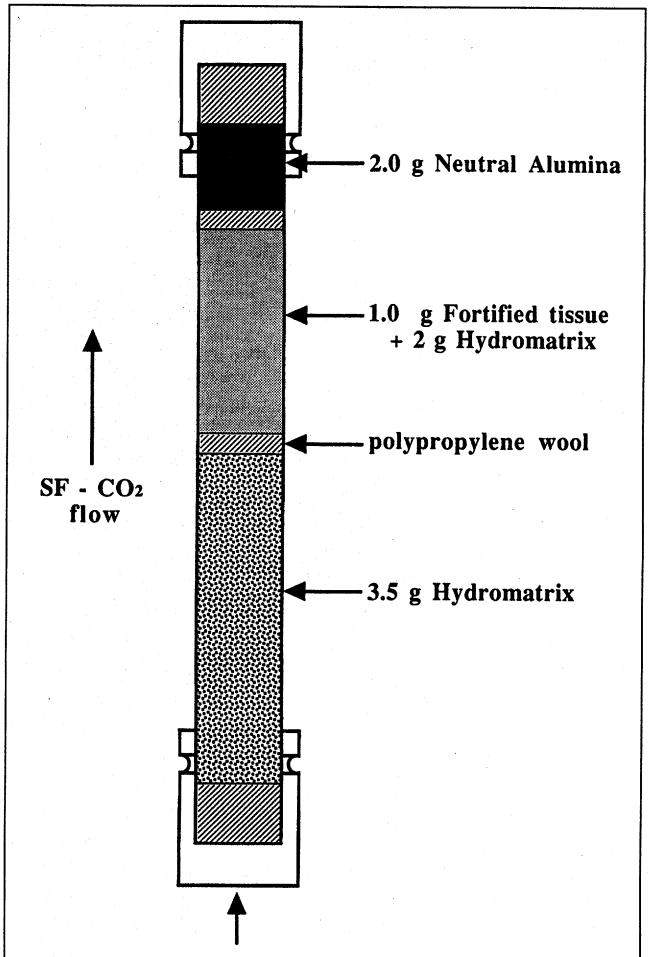

Figure 1. Diagram of an SFE vessel with in-line SPE trap.

of analyte recoveries from tissue was accomplished by comparison of peak heights or areas or both with drug control standards equal to fortification concentrations.

Results and Discussion

To be useful as an analytical tool for drug residues in animal tissues, supercritical fluids must not only extract the analytes from varied tissues but also achieve quantitative recoveries and detections at or below tolerance levels established by the Code of Federal Regulations for edible tissues (6).

In our initial studies on SFE of sulfa drug residues in chicken tissues, parameters similar to those previously established for the successful extraction and recovery of the anti-coccidial nitrobenzamides from chicken tissues were employed (3). The following conditions were used: an extraction temperature of 60°C; a pressure of 10,000 psi (density: 0.993 g/mL); 90 L CO_2 (expanded gas) at a flow rate of 3 L/min; a metering valve temperature of 110°C; and an off-line trap containing 2.0 g neutral alumina. The use of these parameters led to less than satisfactory results for the more polar sulfonamides. Not only were the recoveries unsatisfactory (less than 40–60%), but differences in recoveries between tissues (thigh muscle > breast > liver) were observed. Solvent extraction of the tissue–Hydromatrix mixture after SFE, employing a modification of a previously developed procedure for sulfaquinoxaline and sulfadimethoxine (7), revealed that although less than 60% of the drugs were recovered in the SPE column after depressurization, more than 80% of the drugs had been extracted from the tissues; the difference was apparently "lost" in the system plumbing. An HPLC analysis of a solvent wash of the lines leading from the extraction vessel to the SPE column verified that a portion of the extracted drugs had "precipitated" in the system. In an effort to sweep the analytes from the system, the amount of CO_2 used in the extraction was increased to 120 L, but this led to only a limited increase in and consistency of recoveries. As a result of these observations, studies on in-line trapping of the extracted analytes on neutral alumina were initiated.

In-line trapping of the sulfonamide analytes on 2.0 g of neutral alumina (Figure 1) extracted at 60°C, 10,000 psi (density: 0.993 g/mL), and 3 L CO_2/min for 30 min resulted in overall improved recoveries compared with off-line recovery, but the results remained inconsistent. Subsequent studies showed that channeling of the CO_2 through the loosely packed tissue–Hydromatrix mixture and a slight leakage of the trapped drugs from the alumina trap in the extraction vessel (occurring at 60°C) were the probable reasons. Lowering the oven temperature to 40°C and tightly packing the extraction vessel to limit channeling, as described in the Experimental section, led to satisfactory and consistent recoveries. Moreover, by decreasing the extraction temperature (in effect, increasing the density of the CO_2 from 0.993 to 1.042 g/mL) a higher percentage of the polar analytes was extracted from the matrix.

Table I summarizes, numerically, the observations made during the course of these studies. The results demonstrate the improved recoveries of extracted analytes from the same tissue by in-line versus off-line alumina trapping. The loss of extracted analytes in the system employing off-line trapping may be attributed to one of two occurrences: a decrease in density of the CO_2 resulting from an increase in temperature as the flow approaches the heated metering valve or a deposition in the exit tube of the micrometering valve following decompression of the supercritical fluid CO_2. In-line trapping also corrects the wide differences in recoveries of extracted individual sulfa drugs between tissue types observed with off-line traps. These differences are apparently a result of varying amounts of coextractants (lipids,

Table I. SFE Recoveries of Sulfonamides from Fortified Chicken Tissues*

		Mean % ± SD (n = 5)		
Tissue	SPE†	Sulfamethazine	Sulfadimethoxine	Sulfaquinoxaline
Liver	OL	60.4 ± 3.3	69.3 ± 7.0	54.1 ± 2.5
	IL	89.9 ± 2.3	96.9 ± 1.2	76.4 ± 3.0
Breast	OL	66.7 ± 4.1	79.3 ± 5.6	64.4 ± 5.8
	IL	86.4 ± 2.5	92.0 ± 2.3	75.2 ± 4.1
Thigh	OL	74.9 ± 5.1	83.9 ± 9.5	72.5 ± 7.7
	IL	90.8 ± 3.8	94.8 ± 2.6	79.9 ± 3.2

* The following SFE conditions were used: temperature, 40°C; pressure, 10,000 psi (density, 1.042 g/mL); flow, 3 L/min (expanded gas) for 40 min. The fortification level was 1.0 ppm/sulfa drug.
† Abbreviations: OL, 2.0-g off-line alumina trap; IL, 2.0-g in-line alumina trap.

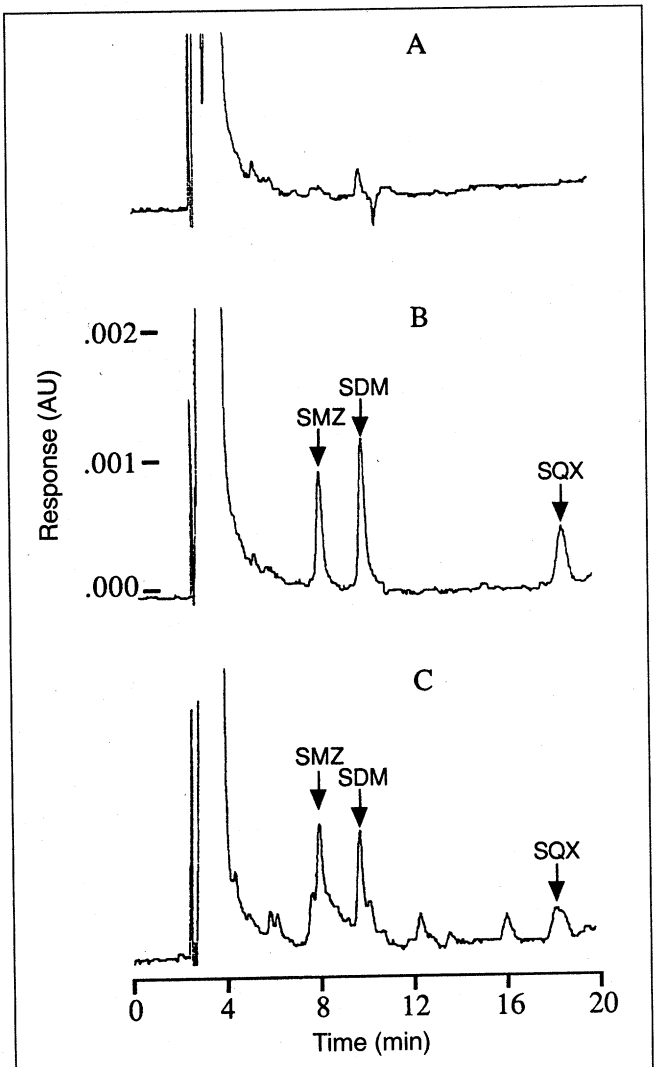

Figure 2. HPLC chromatograms of supercritical fluid extracts (100 µL of 2 mL of SPE eluent) of control liver from in-line SPE (A); liver fortified with 100 ppb sulfonamides from in-line SPE (B); and liver fortified with 100 ppb sulfonamides from off-line SPE.

pigments, etc.) from different tissues and the decrease in density of the CO_2 as the flow approaches the metering valve. Together these factors affect the solubility of the analytes in the supercritical CO_2. With in-line trapping, the vast majority of these coextractants pass through the trap while the sorbent retains the sulfa drugs. It is noteworthy that an 86–91% recovery of sulfamethazine from the tissues was achieved without the use of solvent modifiers. A previous study on sulfamethazine, employing off-line trapping, required 10–25% MeOH as a CO_2 modifier to attain similar results (5). In contrast, the FSIS conventional solvent extraction technique (1) recovers less than 50% of the analyte.

Figure 2 compares HPLC chromatograms of supercritical fluid extracts of chicken livers fortified with 100 ppb of SMZ, SDM, and SQX, which is the tolerance level for each of these compounds in edible tissues (6) using in-line and off-line alumina trapping of analytes. Differences in background levels are the result of contaminating coextractants passing through the in-line trap versus collecting on the off-line trap. The sulfa drugs from the in-line alumina were eluted with the HPLC mobile phase, collected in the first 2 mL of eluent, and required no further cleanup prior to injecting the sample. In contrast, complete elution of the sulfa drugs from the off-line trap required collection of up to 4 mL of eluent; this is presumably the result of eluting solvent and partitioning between coextractants, the majority of which remained on the column. In a study by other investigators on the SFE of sulfa drugs from tissue with CO_2 containing modifiers, an hour-long post-extraction cleanup procedure was required to limit chromatographic background levels (5).

Conclusion

The results of this study demonstrate that SFE with CO_2 at high density in combination with proper sample preparation techniques and in-line trapping on SPE columns represent a promising approach in isolating the polar, nonvolatile sulfa drug residues from animal tissues. Additionally, the SFE–SPE extracts require no post-extraction cleanup and result in HPLC chromatograms with limited background interferences, thereby allowing the detection of residues at less than 100 ppb, which is the current tolerance level established for these drugs in edible tissues.

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Reference to a brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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